Development of Microarrays as a Tool for Discovering Environmental Exposure Indicators

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Toxicogenomics includes research to identify differential gene expression in laboratory and field animals exposed to toxicants, and ultimately, to link the earliest indicators of exposure to adverse effects in organisms and populations. The US EPA National Exposure Research Laboratory has started to develop a multiple aquatic stressor diagnostic exposure model using fathead minnow (*Pimephales promelas*) gene sequences in a microarray platform. Because there is very little gene sequence information for the target organism, an approach involving several different methodologies has been developed to examine specific gene expression and to identify stressor-specific patterns of expression over a portion of the expressed genome. Initially, single exposure-specific microarrays were employed using subtractive cDNA clones to identify differentially expressed genes in minnows exposed to low levels of an estrogenic endocrine disruptor. To complement genes identified in subtractive arrays, other *P. promelas* gene sequences were identified by two techniques. Degenerate PCR primers based on conserved amino acid homology across mammalian and non-mammalian taxa were used to target specific genes of interest such as DNA repair and apoptotic genes. In addition, PCR primers based on a close relative of the fathead minnow, *Cyprinus carpio*, were used to identify homologs of known carp genes. By combining these methods for identifying fathead minnow specific sequences, different functional classes of protein-encoding genes can be used to build an oligo microarray that encompasses a sampling of the expressed genome. Single and multiple exposures will be assessed at the oligo microarray level to gain a better understanding of relative bioavailability of environmental stressors present in mixtures. Thus, the use of microarray technology to examine gene expression promises to be a powerful tool in understanding the impacts of environmental exposures.

METHODS

cDNA Subtraction Libraries

Five adult male fathead minnows were subjected to 72 hour treatment of 17 α-ethynylestradiol at a concentration of 5 ng/L before being euthanized. A similar group was held during this time course with no treatment to serve as a source of control tissues. Livers were harvested and stored in ToTally RNA (Ambion, Austin, TX) solution until the tissues could be homogenized. Poly A+ RNA was directly extracted from 4 livers from each group using Ambion's Micropure Poly A extraction kit.

Using Clontech's (Palo Alto, CA) cDNA subtraction kit, forward and reverse subtractions were performed on adult male livers from control and ethinyl estradiol (EE2) treated fish.

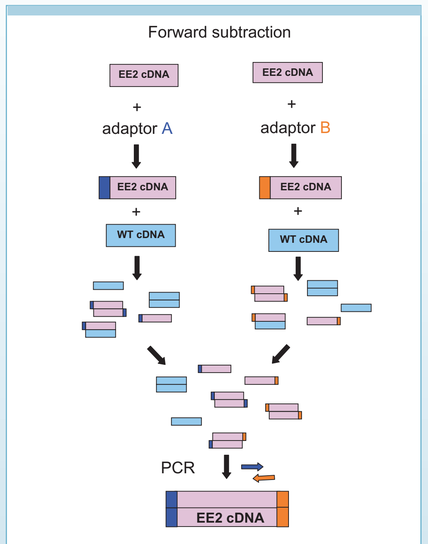

Figure 1. Schematic representation of cDNA subtractive library construction.

Each subtracted library was prepared for microarray printing by isolating individual cDNAs, amplifying each cDNA, and purifying for spotting onto a glass slide. Probe RNA for EE2 was labeled with Cy3 (green) and RNA for WT was labeled with Cy5 (red). Both probes were hybridized to the microarrays to sort out clones highly expressed (upregulated) and downregulated due to EE2 exposure.

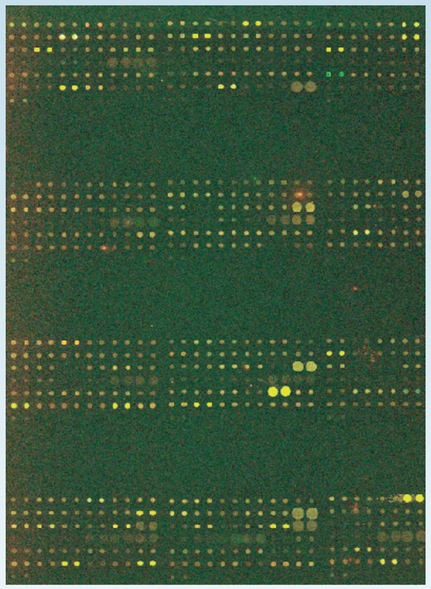

Figure 2. cDNA microarray of EE2 and WT subtracted clones hybridized to EE2 and WT cDNA probes from single individuals.

Gene Homologs

Two separate approaches were used to isolate fathead minnow genes:

- o Degenerate PCR
- o PCR primers based on known genes from a similar fish

Degenerate PCR

Fathead minnow gene homologs of interest were targeted through the use of degenerate PCR primers and a mixture of spleen, liver, and whole 72 hour fry cDNAs. Primers were developed to targeted genes with conserved amino acid sequences. Minimal degeneracy, across several phyla, were key components in the development of these degenerate primers.

Several rounds of PCR were required to isolate potential target amplicons. An initial round of amplification was performed with a low annealing temperature (45° C), followed by a second round of amplification on the electrophoresed size specific gel plug at a higher, more specific annealing temperature (50° C). Resulting size-specific bands were cloned and screened for size before being sequenced with Amersham's (Piscataway, NJ) DYEnamic ET terminator kit.

FATHEAD MINNOW GENE SEQUENCES

degenerate primers, GenBank, carp primers

Structural
12S
18S
28S
alpha-globin
matrix metalloproteinase 9
myosin regulatory light chain
beta-actin
beta-globin
keratin

Metabolic
glucose-6-phosphate dehydrogenase
P450A1
glutathione-S-transferase
PPARb
cytochrome P450 aromatase
cytochrome P450 17alpha-hydroxylase,17,20-lyase
delta-6 fatty acyl desaturase
glucose transporter 1
lactate dehydrongenase I
mt ATP synthase gamma unit
mt cytochrome c oxidase subunit II
mt cytochrome c oxidase subunit III
mt cytochrome b
mt NADH ubiquinone oxidoreductase subunit 4
taurine transporter

Neural
pentraxin
presenilin

Developmental
vitellogenin
zona pellucida glycoprotein
retinol binding protein*

Immunological
macrophage protein
integrin beta-2 chain
myeloid protein

Signal Transduction / Cell Cycle
MAP kinase
G protein-coupled receptor kinase 7
janus kinase 3
MAP kinase kinase 6
jnk b
creatine kinase
mef 2c
myo D
heat shock protein 90 beta
K-ras
Rap1b
wee 1
mdm 2
EF1 alpha
poly(A) polymerase

Endocrine
androgen receptor
inhibin/activin

Carp Primers

Known gene sequences for the common carp were identified in GenBank, and primer sequences were designed for each sequence using OLIGO© 6.58 software (Wojciech Rychlik). Bands of expected size as a result of amplification of a fathead minnow cDNA mix (spleen, liver, and whole 72 hour fry) were extracted and sequenced as described using the amplification primers. Sequences were then checked by BLAST searches for identification.

Fathead Minnow

Pimephales promelas

GOALS

Typical oligonucleotide arrays employ multiple short oligos of 25 bases that correspond to each gene. These oligo sequences are compared against the genome database for that organism to ensure the oligo represents the targeted gene. A series of "control" oligos are also made for each array oligo that incorporate a mismatched nucleotide in order to assess hybridization specificity.

In the absence of whole genome information for the fathead minnow, at least 4 different oligos of 50-70 bases will be constructed for each gene. A set of "control" oligos will also be constructed to accompany the gene specific oligos, and will incorporate 8% mismatched nucleotides.

Example: glutathione-S-transferase

5' GGCCCTGAAGAGGAACCGAGAGAGTCTGGTCGCCGAGCTGAAGCTGTGGGACGGGTACTT 3'
5' GGCCCTGAAGAGGAATCGAGAGAGTCTGATCGCCGGGCTGAAGCTGTGGGACGGATACTT 3'

5' GGGACGGGTACTTGGAGAAGATGGGTAAAGGGTCGTTCCTGGCCGGCAAGAGCTTCACCA 3'
5' GGTACGGGTACTTGGAGAAGATGGGTCAAGGGTCGTTCCTAGCCGGCAAGAGCTTAACCA 3'

5' GGTAAAGGGTCGTTCCTGGCCGGCAAGAGCTTCACCATGGCGGACGTCGTTTGTTTCCCA 3'
5' GGTAAAGGATCGTTCCTGGCCGGCAAGATCTTCACCATGGCGGACGTCATTTGTTGCCCA 3'

5' GGAGAAGATGGGTAAAGGGTCGTTCCTGGCCGGCAAGAGCTTCACCATGGCGGACGTCGT 3'
5' GGAGAGGATGGGTCAAGGGTCGTTCCTGGCCGGCAAGAACTTCACCATGGCGGACTTCGT 3'

Nucleotides indicated in red represent the mismatched bases in the control oligo.

1. Single stressor exposures

Generate single stressor induced gene expression profiles for a number of stressors in the following categories:

- PAHs
- PCBs
- pesticides
- metals
- endocrine disrupting compounds (estrogenic/androgenic)
- perfluorinated organics

2. Diagnostic capability

Pursue any differentially expressed gene for a given exposure as a potential indicator of that stressor. Identify regulatory pathways that appear to be affected and draw on published examination of those pathways in other organisms to infer what probable phenotypic affects might be in progress or what other pathways may be influenced.

3. Mixtures

Examine combinations of stressors to compare/contrast with individual expression patterns for antagonistic or synergistic effects.

Take all identified fathead minnow gene sequences and construct an oligonucleotide array.

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